Published in final edited form as:

Curr Opin Virol. 2020 October ; 44: 26–34. doi:10.1016/j.coviro.2020.06.001.

Reverse genetics approaches for the development of new vaccines against influenza A virus infections

Luis Martinez-Sobrido^{1,&}, Marta L. DeDiego², Aitor Nogales^{3,&}

¹Texas Biomedical Research Institute, San Antonio, TX, 78245, USA

²Department of Molecular and Cell Biology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, 28049 Madrid, Spain

³Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Centro de Investigación en Sanidad Animal (INIA-CISA), 28130 Madrid, Spain

Abstract

Influenza A viruses (IAVs) represent a serious concern globally because they are capable of rapid spread and cause severe disease in humans and other animals. The development and implementation of plasmid-based reverse genetics approaches have allowed the manipulation and recovery of recombinant IAVs from complementary DNA copies of the viral genome. Furthermore, IAV reverse genetics have provided researchers an efficient and powerful platform to introduce specific changes in the viral genome with the final goal of studying IAV biology, designing more effective vaccine strategies, and to reduce the rates of incidence and mortality associated with viral infections. In this review, we briefly discuss IAV reverse genetics and their applications to prevent IAV infections.

Influenza A viruses and their impact on the society

Influenza A viruses (IAVs) are enveloped viruses that belong to the *Orthomyxoviridae* family. Their genomes contain eight negative-sense, single-stranded viral (v)RNA segments which are approximately 12-14 kb in size (Figure 1) [1-3]. On the viral lipid membrane there are the two major glycoproteins, hemagglutinin (HA) and neuraminidase (NA), whose antigenic properties are used to classify IAVs into 18 HA (H1 to H18) and 11 NA (N1 to N11) subtypes [4].

IAV infections have a tremendous impact in human and animal health and the global economy. In humans, IAV infections cause contagious respiratory diseases and substantial morbidity and mortality. Infections can differ in their intensity from mild respiratory disease to pneumonia that can lead to death [5]. The World Health Organization (WHO) estimates

[&]Corresponding authors: Luis Martinez-Sobrido: LMartinez@txbiomed.org, Aitor Nogales: nogales.aitor@inia.es.

Conflict of interest statement

The authors declare no conflict of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

that, in humans, seasonal IAV infections are responsible for ~5 million cases of severe disease and ~500,000 annual deaths ([https://www.who.int/news-room/fact-sheets/detail/influenza-\(seasonal\)](https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal))). Nowadays, two subtypes of IAV (H1N1 and H3N2) co-circulate in humans, causing seasonal epidemics [6]. However, because IAVs are maintained in a wide range of animal species, they have the potential to origin pandemics as occurred when a swine-origin H1N1 IAV (pH1N1) emerged in humans in 2009, spreading rapidly and infecting thousands of individuals worldwide [7]. In addition, avian influenza viruses (AIV) H5, H7, and H9 subtypes are able to cross the species barrier to infect humans. Importantly, highly pathogenic AIVs (HPAIVs), such as H5N1 that kill 90-100% of infected domestic flocks, have been responsible for hundreds of human infections, resulting in severe disease and mortality rates of up to 50% [8]. Moreover, low pathogenic AIVs (LPAIVs), as H9N2, can cause pandemics and also donate internal genes to generate novel human-pandemic reassortants [4,8]. Antigenic drift and antigenic shift are the two major mechanisms responsible for the emergence of seasonal and pandemic IAVs, respectively [4]. In the first case, mutations in the viral genome are introduced and selected in the viral population. Antigenic shift is a consequence of the co-infection of a cell with two or more IAV strains and the generation of new viruses containing reassorted genomes. Either antigenic drift or shift could lead to the generation of new viruses with increased viral fitness, drug-resistance or different antigenic characteristics.

Current vaccines to combat IAV infections

Vaccination is the main option for the control of IAV infections. However, current vaccination strategies to prevent IAV infections are only partially effective [9]. Therefore, the development of novel prophylactic approaches, including universal vaccines, are highly desired and required for the efficient control of IAV infections. Given the genome variability of IAV glycoproteins HA and NA, the two major proteins inducing protective neutralizing antibody responses, viruses with new antigenic proteins emerge constantly every year [6,10,11]. Hence, seasonal vaccines must be updated annually to assure that the viral HA and NA glycoproteins in the vaccine match circulating strains [6,10,11]. Importantly, seasonal vaccines are usually ineffective against pandemic IAV strains [9]. To avoid these concerns, there is currently a high public health interest to develop broadly protective, universal, IAV vaccines which ideally, confer cross-protection against all seasonal and potentially pandemic IAVs [12].

To control seasonal IAV infection in the human population, there are three mayor kinds of vaccines, including recombinant viral HA (rHA), inactivated influenza vaccines (IIV) and live-attenuated influenza vaccines (LAIV) (Figure 2). Regardless of the vaccine type, seasonal vaccines contain antigens from the three (trivalent) or four (quadrivalent) circulating influenza virus strains: two IAV subtypes (H1N1 and H3N2) and one (trivalent) or two (quadrivalent) influenza B virus (IBV) subtypes (Victoria-like or Yamagata-like lineage) [6,10]. Vaccines developed against pandemic strains or for the prevention of IAV infections in domestic animal species such as poultry, swine, dogs, or horses are also based in one of the indicated types of vaccine [6,10]. For seasonal human vaccines, the strategy to produce IIVs and LAIVs involve the co-infection of embryonated chicken eggs with the WHO candidate IAV strain and a Master Donor Virus (MDV) (Figure 2) [6,10]. The

segmented genome of IAV allows for the generation of a reassortant virus containing the surface glycoproteins (HA and NA) of the WHO recommended strain and the six remaining internal genes of the MDV. Then, the new vaccine strain is selected using neutralizing antibodies against the HA and NA proteins of the MDV. The MDV that is used in the United States to produce the IAV LAIV is A/Ann Arbor/6/60 H2N2 that contains four mutations (PB2: N265S; PB1: K391E, E581G, and A661T; and NP: D34G) conferring a cold adapted (ca), temperature sensitive (ts) and attenuated (att) phenotype, which allow the vaccine strain to replicate at low (25 °C to 33 °C) temperatures, but limit viral replication at higher (37 °C or 39 °C) temperatures [2,6,10]. Although the mechanism of attenuation for the LAIV has not been completely determined, multiple variables could be involved. Importantly, the LAIV provide important advantages compared with rHA or IIV, such as mimicking natural viral exposure to provide local immunity, the induction of broad immunity (including antibody and cellular responses), long-term protection, and greater levels of cross-protection against drifted viral variants. On the other hand, IIV or rHA vaccines induce mainly protective humoral responses with limited, if any, cross protection efficacy against drifted IAV strains [6,13]. However, the current LAIV is only recommended for immunocompetent parts of the population, which represents an important drawback to control IAV infections in vulnerable individuals.

Reverse genetics approaches for the generation of IAV vaccines

IAV reverse genetics technologies that allow the generation of recombinant viruses from complementary (c)DNAs have transformed the field, and have made possible the study of the underlying mechanisms of viral pathogenicity, transmission or interaction with host factors to expand our knowledge of IAV infections [6]. Moreover, reverse genetics have been important for the development of novel vaccine approaches against IAV infections and for the identification of antivirals. The most extended plasmid-based reverse genetics techniques for IAV allows for the simultaneous expression, using an ambisense coding strategy, of the viral replication machinery and the viral (v)RNAs in susceptible cells transfected with eight plasmids (Figure 3). Ambisense plasmids are made of the polymerase I (Pol I) cassette that includes the RNA Pol I promoter and terminator sequences that initiates and terminates transcription, allowing the synthesis of vRNAs. Although Pol I promoter is species specific, the promoters of different species have been identified and used for the rescue of recombinant IAVs in other cell lines, including, among others, avian, canine, equine or murine cells [14]. Ambisense plasmids also contain, in opposite orientation to the Pol I cassette, the polymerase II (Pol II) cassette made of a Pol II promoter, such as the chicken β -actin or the cytomegalovirus (CMV) promoter, which ensure robust levels of protein expression from the same viral cDNA, and a polyadenylation sequence, such as the bovine growth hormone (BGH). Transfection of susceptible cells with these 8 ambisense plasmids made possible the recovery of recombinant IAVs from the supernatants of transfected cells (Figure 3) [14].

Novel approaches to generate LAIV based on reverse genetics

The implementation of plasmid-based reverse genetics techniques for the generation of recombinant IAVs has been one of the most significant advances in the field of IAV research.

These procedures have been key for the development of novel LAIV approaches to prevent and control IAV infections in humans and other animal species [6]. In this review, we highlight novel approaches that have been recently described for the development of LAIVs for the treatment of IAV infections (Figure 4).

Novel MDVs

Currently, A/Ann Arbor/6/60 H2N2 or A/Leningrad/134/17/57 H2N2 viruses are the two MDV backbones that are being used to create seasonal LAIVs against IAV infections in the United States and Russia, respectively. The amino acid changes responsible for the ts, ca and att phenotypes of these MDVs have been described previously [6,10,15-17]. The potential reasons for the reduced effectiveness of seasonal LAIVs during the last years, have not been entirely understood and multiple variables could be involved [18]. It has been suggested, as one potential cause, that current MDVs that provide the six internal gene segments (PB2, PB1, PA, NP, M and NS), are based on strains that significantly diverge from those circulating viruses, limiting an effective and protective cellular immune response [19]. Thus, the development of a LAIV platform based in contemporary MDV strains, where the internal viral gene segments better match those of circulating strains, could represent a better approach for the treatment of seasonal, and potentially pandemic, IAV infections (Figure 4A). Importantly, it has been reported that introduction of the ts, ca, att signature of both the United States A/Ann Arbor/6/60 H2N2 and the Russian A/Leningrad/134/17/57 H2N2 MDVs in the background of other viral strains results in similar ts, ca and att phenotype, demonstrating the feasibility of implementing this approach to improve protective immunity, as compared to the conventional LAIV MDVs [16].

NS1 deficient or truncated LAIVs

IAV non-structural 1 (NS1) protein has the ability to counteract the host innate immune response and hijack the antiviral responses during viral infection [20,21]. Therefore, multiple LAIV strategies based on the deletion and/or partial truncation of NS1 have been tested for avian [22-26], canine [27], equine [28], swine [29-32], and human [33-35] IAVs (Figure 4B). A correlation between the length of the NS1 protein and viral fitness and/or attenuation, most likely due to the presence of multiple functional domains in NS1, has been reported. Importantly, NS1 mutant viruses are considered as very promising LAIVs, given that their replication is highly affected in interferon (IFN)-competent hosts, while they are able to induce a protective immune response against wild-type forms of the virus when administrated into mice [27,34], pigs [29-31], horses [28], birds [22-24], macaques [35] or humans [33].

Altered NS1 and/or PA-X LAIVs

The PA-X and NS1 proteins of IAV are virulence factors that modulate the host innate immune response upon infection allowing viral replication. In addition, both proteins share the ability to induce cellular shutoff using different mechanisms [20]. Recent evidences have shown that these two viral factors could evolve together to maintain the required equilibrium in the inhibition of host gene expression [36]. The functional activity of PA-X and NS1 in

modulating antiviral responses has been altered to generate novel LAIVs with different levels of immunogenicity or safety profiles (Figure 4C) [37,38]. For instance, we assessed a set of recombinant pH1N1 viruses containing the ts, ca, att signature of the MDV A/Ann Arbor/6/60 H2N2 and encoding PA-X and NS1 proteins with different abilities to inhibit gene expression [37]. Lower levels of viral replication and humoral responses were observed in mice inoculated with viruses where both proteins have, or do not have, the capability to inhibit host gene expression [37]. Recently, to improve the safety profile of the MDV A/Ann Arbor/6/60 H2N2 and make it available to individuals currently excluded for its use, we modulated the ability of the NS1 and/or PA-X proteins in the MDV to inhibit host gene expression [38]. Our data demonstrate that eliminating the ability of PA-X to inhibit host gene expression, limits the replication of the MDV *in vivo*, while maintaining high levels of protective efficacy [38], suggesting that altering inhibition of host gene expression by NS1 and/or PA-X could be used to vaccinate individuals excluded for the use of the current LAIV.

Synonymous genome recoding for the development of LAIVs

The flexibility of the genetic code where different amino acids can be encoded by more than one codon [39], which are not equally represented in the genomes, has opened the possibility to recode viral genomes, to create a new generation of LAIVs (Figure 4D). Because IAV and other viruses need to hijack the cellular machinery to replicate, IAV has optimized its genome to the host codon usage [40,41]. Taking advantage of synthetic biology and reverse genetics approaches, several LAIVs have been generated by synonymous genome recoding using a codon-pair [42-44] or a codon bias [45] deoptimization strategy to decrease expression levels of different IAV proteins [46]. Importantly codon deoptimization (CD) of the viral genome provides multiple advantages for LAIV design and implementation including, among others: 1) the level of attenuation depends of the gene(s) deoptimized and the sequence length changed; 2) CD IAVs maintain the immunogenic properties of their parental strains but with high levels of attenuation; 3) CD IAVs displayed similar levels of replication than parental viruses in some cell lines, including Food and Drug Administration (FDA)-approved cells for vaccine manufacture and production [42-46]; and, 4) a single vaccination with CD IAVs results in protective immune responses against lethal challenges with wild-type viruses [42-46]. Importantly, non-coding regions (NCRs) and/or packaging signals cannot be recoded because of their key role in viral replication and genome encapsulation, respectively.

LAIVs based on rearrangement of the viral genome

Reverse genetics techniques have allowed the rearrangement of the IAV genome with the goal to generate new LAIVs. These include engineered recombinant viruses containing modified M and/or NS segments (Figure 4E). In the WT viruses, IAV M and NS segments express two different viral proteins (M1 and M2 or NS1 and NEP, respectively) using an alternative splicing mechanism (Figure 1) [1,3]. Separating the overlapping open reading frame (ORFs) of the M1/M2 (M segment) and/or the NS1/NEP proteins (NS segment) using the porcine teschovirus 1 (PTV-1) 2A autocleavage site has allowed the generation of non-splicing viruses (Figure 4E) [47]. IAVs harboring the modified M segment (alone or in

combination with modified NS segment) were highly attenuated in mice, but able to provide, upon a single immunization, complete protection against a lethal viral challenge [48]. An alternative strategy was used to generate a bivalent LAIV against H5N1 and H9N2 AIVs (Figure 4F) [49]. In this case, the NEP was relocated to the PB1 segment and the H5 ORF was cloned downstream of a NS1 truncated (1-99) NS segment [49]. This system allows the co-linear expression of H9N2 PB1 and NEP from the PB1 segment, and H5N1 HA and NS1 1-99 from the NS segment. Importantly, the rearranged H9N2 IAV expressing H5N1 HA was able to induce protection against challenge with H9N2 and H5N1 IAVs in mice and ferrets [49]. Furthermore, HA and NA viral segments were also modified to generate bivalent LAIVs against H1 and H3 IAVs, which could be useful for the development of new seasonal LAIVs (Figure 4F) [50].

Single cycle infectious IAV (sciIAV) LAIVs

SciIAVs containing substitutions or deletions of essential viral genes required for the completion of IAV life cycle have been described as feasible LAIVs for the treatment of IAV infection (Figure 4G) [51-53]. In order to propagate sciIAVs, the lack of the viral gene must be compensated, *in trans*, by stable cell lines providing the missing viral protein [52,53]. With the implementation of reverse genetics approaches, multiple sciIAVs have been generated, including those deficient in PB2, PB1, PA, HA or NA viral genes [52,53,62-66,54-61]. These sciIAVs have shown to be useful to study viral sequences and mechanisms involved in viral genome encapsidation [61,67]; the identification of host factors important for viral replication, novel antivirals or neutralizing antibodies [51,53,68]; to reduce biosafety requirements to work with HPAIV strains [51]; and for their implementation as safe, immunogenic and protective LAIVs [54-59,62-65]. Importantly, sciIAVs are safer than LAIVs because their replication is limited to one cycle in parental cells, but are more immunogenic than IIV, since they can replicate to one cycle [52].

Premature termination codon (PTC) LAIVs

Recombinant viruses containing in their genome multiple premature termination codons (PTCs) distributed in different viral segments have also been described as a novel LAIV (Figure 4H) [69]. In this approach, a transgenic cell line susceptible to IAV replication that also contained the orthogonal translation machinery, assures that viruses could be propagated only in this cell line, while they are replication-incompetent in parental cells and/or *in vivo* [69]. Notably, these PTC IAVs have been shown to be good LAIV candidates in different animal models of infection, including mice, guinea pigs, and ferrets. Importantly, PTC IAVs were able to induce robust humoral and cellular responses, which protected animals against both homosubtypic and heterosubtypic IAV challenges [69]. Moreover, these PTC IAVs were genetically stable, further demonstrating their feasibility as a novel LAIV for the treatment of IAV infections.

Conclusion and future directions

IAV infections represent a serious treat for public health and worldwide economy. Although vaccination is the most effective strategy to prevent and control IAV infections, the

effectiveness of current IAV vaccine approaches is suboptimal [18]. The implementation of reverse genetics systems to generate recombinant IAVs have provided with the opportunity to develop novel and more effective LAIVs to protect humans and other animal species against IAV infections [6,10]. In this review, we have briefly discussed some of the latest approaches for the development and implementation of new LAIVs for the treatment of IAV infections. New LAIV approaches, including those for the development of universal vaccines against IAVs [12], providing more robust, long-lasting immune responses, are clearly guarantee in the near future. In addition, combination of several attenuation markers on multiple viral segments is expected to increase the safety, without affecting protective immunogenicity, of future LAIVs. However, attention should be pay to genetic stability of these new LAIV candidates. Based on the number of new LAIV approaches developed during the last years, it is expected that more clinical trials will be launched in the near future.

Acknowledgments

We want to thank all the work conducted by the influenza researchers' referenced in this manuscript that have contributed significantly to both the implementation of influenza reverse genetics techniques and/or in the development of the novel approaches for the development of LAIVs. We want to apologize if we omitted any manuscript(s) describing a new approach. Influenza research in LM-S laboratory has been partially funded by the New York Influenza Center of Excellence (NYICE, NIH 272201400005C), a member of the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Department of Health and Human Services, Centers of Excellence for Influenza Research and Surveillance (CEIRS) contract No. HHSN272201400005C (NYICE), the NIH/NIAID 1R01AI145332, and the Department of Defense (DoD) Peer Reviewed Medical Research Program (PRMRP) grant W81XWH-18-1-0460. Research in M.L.D laboratory was supported with funds from Comunidad de Madrid, Spain, reference 2017-T1/BMD-5155 and the Spanish Ministry of Science, Innovation and Universities (RTI-2018-094213-A-I00). Research in A.N laboratory was partially funded with the "Ramon y Cajal" Fellowship RYC-2017 from Spanish Ministry of Economy, Industry and Competitiveness and the Spanish Ministry of Science, Innovation and Universities (AGL2017-82570-R).

Abbreviations

AIV	Avian influenza virus
Att	Attenuated
BGH	Bovine growth hormone
Ca	Cold-adapted
CD	Codon deoptimized
cDNA	complementary DNA
CMV	Cytomegalovirus
FDA	Food and drug administration
GFP	green fluorescent protein
HA	Hemagglutinin
HI	hemagglutination inhibition
HPAIV	Highly pathogenic avian influenza virus(es)

IAV	Influenza A virus
IBV	Influenza B virus
IFN	Interferon
IIV	Influenza inactivated vaccine
ISGs	Interferon (IFN)-stimulated genes
LAIV	Live-attenuated influenza vaccine
LPAIV	Low pathogenic avian influenza virus(es)
M1	Matrix protein 1
M2	Matrix protein 2
MDV	Master donor virus
Ms	M split
NA	Neuraminidase
NAbs	Neutralizing antibodies
NCR	Non-coding region
NEP	Nuclear export protein
NP	Nucleoprotein
NS1	Non-structural protein 1
NSs	NS split
ORF	Open reading frame
PA	Polymerase acid
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PFU	Plaque forming units
pH1N1	pandemic H1N1 virus
Pol I	Polymerase I
Pol II	Polymerase II
PTC	premature termination codon
PTV-1	porcine teschovirus 1
RdRp	RNA-dependent RNA polymerase

rHA	recombinant hemagglutinin
sciIAV	single-cycle infectious Influenza A Virus
ssRNA	single-stranded RNA
Ts	Temperature-sensitive
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein
WT	Wild-type
WHO	World Health Organization

References

1. Shaw PMP: Orthomyxoviridae: the viruses and their replication. **In:** Knipe DM.; Howley PM; Griffin DE; Lamb RA; Martin MA, **editors.** . Fields Virol 5th Lippincott Williams Wilkins PA, USA 2007., **In:**, **editors.**
2. Martinez-Sobrido L, Peersen O, Nogales A: Temperature Sensitive Mutations in Influenza A Viral Ribonucleoprotein Complex Responsible for the Attenuation of the Live Attenuated Influenza Vaccine. *Viruses* 2018, 10.
3. Dou D, Revol R, Östbye H, Wang H, Daniels R: Influenza A virus cell entry, replication, virion assembly and movement. *Front Immunol* 2018, doi:10.3389/fimmu.2018.01581.
4. Wille M, Holmes EC: The Ecology and Evolution of Influenza Viruses. *Cold Spring Harb Perspect Med* 2019, doi:a038489 [pii]10.1101/cshperspect.a038489cshperspect.a038489 [pii].
5. Garten R, Blanton L, Elal AIA, Alabi N, Barnes J, Biggerstaff M, Brammer L, Budd AP, Burns E, Cummings CN, et al.: Update: Influenza Activity in the United States During the 2017-18 Season and Composition of the 2018-19 Influenza Vaccine. *MMWR Morb Mortal Wkly Rep* 2018, 67:634–642. [PubMed: 29879098]
6. Blanco-Lobo P, Nogales A, Rodriguez L, Martinez-Sobrido L: Novel Approaches for The Development of Live Attenuated Influenza Vaccines. *Viruses* 2019, 11.
7. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, et al.: Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* (80-) 2009, 325:197–201.
8. Sutton TC: The Pandemic Threat of Emerging H5 and H7 Avian Influenza Viruses. *Viruses* 2018, 10.
9. McLean HQ, Caspard H, Griffin MR, Poehling KA, Gaglani M, Belongia EA, Talbot HK, Peters TR, Murthy K, Ambrose CS: Effectiveness of live attenuated influenza vaccine and inactivated influenza vaccine in children during the 2014-2015 season. *Vaccine* 2017, 35:2685–2693. [PubMed: 28408121]
10. Nogales A, Martinez-Sobrido L: Reverse Genetics Approaches for the Development of Influenza Vaccines. *Int J Mol Sci* 2016, 18.
11. Clark AM, DeDiego ML, Anderson CS, Wang J, Yang H, Nogales A, Martinez-Sobrido L, Zand MS, Sangster MY, Topham DJ: Antigenicity of the 2015-2016 seasonal H1N1 human influenza virus HA and NA proteins. *PLoS One* 2017, 12:e0188267. [PubMed: 29145498]
12. Rajao DS, Perez DR: Universal Vaccines and Vaccine Platforms to Protect against Influenza Viruses in Humans and Agriculture. *Front Microbiol* 2018, 9:123. [PubMed: 29467737]
13. Sridhar S, Brokstad KA, Cox RJ: Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines. *Vaccines (Basel)* 2015, 3:373–389. [PubMed: 26343192]

14. Nogales A, Rodriguez L, Chauche C, Huang K, Reilly EC, Topham DJ, Murcia PR, Parrish CR, Martinez-Sobrido L: Temperature-Sensitive Live-Attenuated Canine Influenza Virus H3N8 Vaccine. *J Virol* 2017, 91.
15. Cox NJ, Kitame F, Kendal AP, Maassab HF, Naeve C: Identification of sequence changes in the cold-adapted, live attenuated influenza vaccine strain, A/Ann Arbor/6/60 (H2N2). *Virology* 1988, 167:554–567. [PubMed: 2974219]
16. Smith A, Rodriguez L, Ghouayel ME, Nogales A, Chamberlain JM, Sortino K, Reilly E, Feng C, Topham DJ, Martinez-Sobrido L, et al.: A live-attenuated influenza vaccine (LAIV) elicits enhanced heterologous protection when the internal genes of the vaccine are matched to the challenge virus. *J Virol* 2019, doi:JV1.01065-19 [pii]10.1128/JVI.01065-19.*The findings from this work suggest that it may be possible to improve the efficacy of LAIVs by better matching the sequence of the MDV to currently circulating strains.
17. Rodriguez L, Blanco-Lobo P, Reilly EC, Maehigashi T, Nogales A, Smith A, Topham DJ, Dewhurst S, Kim B, Martinez-Sobrido L: Comparative Study of the Temperature Sensitive, Cold Adapted and Attenuated Mutations Present in the Master Donor Viruses of the Two Commercial Human Live Attenuated Influenza Vaccines. *Viruses* 2019, 11.**This study supports the feasibility of using a different virus backbone as MDV for the development of improved LAIVs for the prevention of IAV infections.
18. Caspard H, Mallory RM, Yu J, Ambrose CS: Live-Attenuated Influenza Vaccine Effectiveness in Children From 2009 to 2015–2016: A Systematic Review and Meta-Analysis. *Open Forum Infect Dis* 2017, doi:10.1093/ofid/ofx111.
19. Ambrose CS, Bright H, Mallory R: Letter to the editor: Potential causes of the decreased effectiveness of the influenza A(H1N1)pdm09 strain in live attenuated influenza vaccines. *Euro Surveill* 2016, 21.
20. Nogales A, Martinez-Sobrido L, Topham DJ, DeDiego ML: Modulation of Innate Immune Responses by the Influenza A NS1 and PA-X Proteins. *Viruses* 2018, 10.
21. Clark AM, Nogales A, Martinez-Sobrido L, Topham DJ, DeDiego ML: Functional Evolution of Influenza Virus NS1 Protein in Currently Circulating Human 2009 Pandemic H1N1 Viruses. *J Virol* 2017, 91.
22. Steel J, Lowen AC, Pena L, Angel M, Solorzano A, Albrecht R, Perez DR, Garcia-Sastre A, Palese P: Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. *J Virol* 2009, 83:1742–1753. [PubMed: 19073731]
23. Wang L, Suarez DL, Pantin-Jackwood M, Mibayashi M, Garcia-Sastre A, Saif YM, Lee CW: Characterization of influenza virus variants with different sizes of the non-structural (NS) genes and their potential as a live influenza vaccine in poultry. *Vaccine* 2008, 26:3580–3586. [PubMed: 18539366]
24. Choi EH, Song MS, Park SJ, Pascua PN, Baek YH, Kwon HI, Kim EH, Kim S, Jang HK, Poo H, et al.: Development of a dual-protective live attenuated vaccine against H5N1 and H9N2 avian influenza viruses by modifying the NS1 gene. *Arch Virol* 2015, 160:1729–1740. [PubMed: 25959557]
25. Jang H, Ngunjiri JM, Lee CW: Association between Interferon Response and Protective Efficacy of NS1-Truncated Mutants as Influenza Vaccine Candidates in Chickens. *PLoS One* 2016, 11:e0156603. [PubMed: 27257989]
26. Jang H, Elaish M, Kc M, Abundo MC, Ghorbani A, Ngunjiri JM, Lee CW: Efficacy and synergy of live-attenuated and inactivated influenza vaccines in young chickens. *PLoS One* 2018, 13:e0195285. [PubMed: 29624615]
27. Nogales A, Huang K, Chauche C, DeDiego ML, Murcia PR, Parrish CR, Martinez-Sobrido L: Canine influenza viruses with modified NS1 proteins for the development of live-attenuated vaccines. *Virology* 2017, 500:1–10. [PubMed: 27750071] *This is the first description of a LAIV for the prevention of H3N8 CIV in dogs. Moreover, immunogenicity and protection efficacy was better than that observed with a commercial IIV.
28. Quinlivan M, Zamarin D, Garcia-Sastre A, Cullinane A, Chambers T, Palese P: Attenuation of equine influenza viruses through truncations of the NS1 protein. *J Virol* 2005, 79:8431–8439. [PubMed: 15956587]

29. Vincent AL, Ma W, Lager KM, Janke BH, Webby RJ, Garcia-Sastre A, Richt JA: Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine. *Vaccine* 2007, 25:7999–8009. [PubMed: 17933442]
30. Richt JA, Lekcharoensuk P, Lager KM, Vincent AL, Loiacono CM, Janke BH, Wu WH, Yoon KJ, Webby RJ, Solorzano A, et al.: Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. *J Virol* 2006, 80:11009–11018. [PubMed: 16943300]
31. Solorzano A, Webby RJ, Lager KM, Janke BH, Garcia-Sastre A, Richt JA: Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. *J Virol* 2005, 79:7535–7543. [PubMed: 15919908]
32. Kappes MA, Sandbulte MR, Platt R, Wang C, Lager KM, Henningson JN, Lorusso A, Vincent AL, Loving CL, Roth JA, et al.: Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs. *Vaccine* 2012, 30:280–288. [PubMed: 22067263]
33. Wacheck V, Egorov A, Groiss F, Pfeiffer A, Fuereder T, Hoeflmayer D, Kundi M, Popow-Kraupp T, Redlberger-Fritz M, Mueller CA, et al.: A novel type of influenza vaccine: safety and immunogenicity of replication-deficient influenza virus created by deletion of the interferon antagonist NS1. *J Infect Dis* 2010, 201:354–362. [PubMed: 20039806]
34. Pica N, Langlois RA, Krammer F, Margine I, Palese P: NS1-truncated live attenuated virus vaccine provides robust protection to aged mice from viral challenge. *J Virol* 2012, 86:10293–10301. [PubMed: 22787224]
35. Baskin CR, Bielefeldt-Ohmann H, Garcia-Sastre A, Tumpey TM, Van Hoeven N, Carter VS, Thomas MJ, Proll S, Solorzano A, Billharz R, et al.: Functional genomic and serological analysis of the protective immune response resulting from vaccination of macaques with an NS1-truncated influenza virus. *J Virol* 2007, 81:11817–11827. [PubMed: 17715226]
36. Nogales A, Martinez-Sobrido L, Chiem K, Topham DJ, DeDiego ML: Functional Evolution of the 2009 Pandemic H1N1 Influenza Ns1 and Pa in Humans. *J Virol* 2018, 92.**This study demonstrates the need to monitor the evolution of pH1N1 in humans for mutations in the viral genome that could result in enhanced virulence. Importantly, these results further support previous findings suggesting that inhibition of host gene expression mediated by IAV NS1 and PA-X proteins is subject to a balance which can determine virus pathogenesis and fitness.
37. Nogales A, Rodriguez L, DeDiego ML, Topham DJ, Martinez-Sobrido L: Interplay of PA-X and NS1 Proteins in Replication and Pathogenesis of a Temperature-Sensitive 2009 Pandemic H1N1 Influenza A Virus. *J Virol* 2017, 91.
38. Hilimire TA, Nogales A, Chiem K, Ortego J, Martinez-sobrido L: Increasing the safety profile of the master donor live attenuated influenza vaccine. *Pathogens* 2020, doi:10.3390/pathogens9020086.**This work demonstrates a broadly applicable strategy of tuning the amount of host antiviral responses induced by the IAV MDV for the development of a safer LAIV. Moreover, results also demonstrate, for the first time, the feasibility of genetically manipulating the backbone of the IAV MDV to improve the efficacy of the current IAV LAIV.
39. Plotkin JB, Kudla G: Synonymous but not the same: the causes and consequences of codon bias. *Nat Rev Genet* 2011, 12:32–42. [PubMed: 21102527]
40. Goni N, Iriarte A, Comas V, Sonora M, Moreno P, Moratorio G, Musto H, Cristina J: Pandemic influenza A virus codon usage revisited: biases, adaptation and implications for vaccine strain development. *Virol J* 2012, 9:263. [PubMed: 23134595]
41. Bahir I, Fromer M, Prat Y, Linial M: Viral adaptation to host: a proteome-based analysis of codon usage and amino acid preferences. *Mol Syst Biol* 2009, 5:311. [PubMed: 19888206]
42. Yang C, Skiena S, Futcher B, Mueller S, Wimmer E: Deliberate reduction of hemagglutinin and neuraminidase expression of influenza virus leads to an ultraprotective live vaccine in mice. *Proc Natl Acad Sci U S A* 2013, 110:9481–9486. [PubMed: 23690603]
43. Mueller S, Coleman JR, Papamichail D, Ward CB, Nimnual A, Futcher B, Skiena S, Wimmer E: Live attenuated influenza virus vaccines by computer-aided rational design. *Nat Biotechnol* 2010, 28:723–726. [PubMed: 20543832]
44. Broadbent AJ, Santos CP, Anafu A, Wimmer E, Mueller S, Subbarao K: Evaluation of the attenuation, immunogenicity, and efficacy of a live virus vaccine generated by codon-pair bias de-

- optimization of the 2009 pandemic H1N1 influenza virus, in ferrets. *Vaccine* 2016, 34:563–570. [PubMed: 26655630]
45. Nogales A, Baker SF, Ortiz-Riano E, Dewhurst S, Topham DJ, Martinez-Sobrido L: Influenza A virus attenuation by codon deoptimization of the NS gene for vaccine development. *J Virol* 2014, 88:10525–10540. [PubMed: 24965472]
 46. Baker SF, Nogales A, Martinez-Sobrido L: Downregulating viral gene expression: codon usage bias manipulation for the generation of novel influenza A virus vaccines. *Futur Virol* 2015, 10:715–730.
 47. Nogales A, Ávila-Pérez G, Rangel-Moreno J, Chiem K, DeDiego ML, Martínez-Sobrido L: A Novel Fluorescent and Bioluminescent Bireporter Influenza A Virus To Evaluate Viral Infections. *J Virol* 2019, doi:10.1128/jvi.00032-19.*This work describes, for the first time, a recombinant replication-competent bireporter IAV (BIRFLU) that stably expresses two reporter genes (one fluorescent and one luciferase) to track IAV infections *in vitro* and *in vivo*.
 48. Nogales A, DeDiego ML, Topham DJ, Martinez-Sobrido L: Rearrangement of Influenza Virus Spliced Segments for the Development of Live-Attenuated Vaccines. *J Virol* 2016, 90:6291–6302. [PubMed: 27122587] ** This work describe a novel approach to generate safer and more efficient LAIVs based on recombinant viruses whose genomes encode non-overlapping and independent M1/M2 or both M1/M2 and NS1/NEP open reading frames. Moreover, this is the first time in which an influenza virus containing a restructured M segment has been described.
 49. Pena L, Sutton T, Chockalingam A, Kumar S, Angel M, Shao H, Chen H, Li W, Perez DR: Influenza viruses with rearranged genomes as live-attenuated vaccines. *J Virol* 2013, 87:5118–5127. [PubMed: 23449800]
 50. Lu B, Zhou H, Ye D, Kemble G, Jin H: Improvement of influenza A/Fujian/411/02 (H3N2) virus growth in embryonated chicken eggs by balancing the hemagglutinin and neuraminidase activities, using reverse genetics. *J Virol* 2005, 79:6763–6771. [PubMed: 15890915]
 51. Martinez-Sobrido L, Cadagan R, Steel J, Basler CF, Palese P, Moran TM, Garcia-Sastre A: Hemagglutinin-pseudotyped green fluorescent protein-expressing influenza viruses for the detection of influenza virus neutralizing antibodies. *J Virol* 2010, 84:2157–2163. [PubMed: 19939917]
 52. Nogales A, Baker SF, Domm W, Martinez-Sobrido L: Development and applications of single-cycle infectious influenza A virus (sciIAV). *Virus Res* 2016, 216:26–40. [PubMed: 26220478]
 53. Baker SF, Nogales A, Santiago FW, Topham DJ, Martinez-Sobrido L: Competitive detection of influenza neutralizing antibodies using a novel bivalent fluorescence-based microneutralization assay (BiFMA). *Vaccine* 2015, 33:3562–3570. [PubMed: 26044496]
 54. Sarawar S, Hatta Y, Watanabe S, Dias P, Neumann G, Kawaoka Y, Bilsel P: M2SR, a novel live single replication influenza virus vaccine, provides effective heterosubtypic protection in mice. *Vaccine* 2016, 34:5090–5098. [PubMed: 27595896]
 55. Hatta Y, Boltz D, Sarawar S, Kawaoka Y, Neumann G, Bilsel P: M2SR, a novel live influenza vaccine, protects mice and ferrets against highly pathogenic avian influenza. *Vaccine* 2017, 35:4177–4183. [PubMed: 28668565]
 56. Masic A, Pyo HM, Babiuk S, Zhou Y: An eight-segment swine influenza virus harboring H1 and H3 hemagglutinins is attenuated and protective against H1N1 and H3N2 subtypes in pigs. *J Virol* 2013, 87:10114–10125. [PubMed: 23843633]
 57. Guo H, Baker SF, Martinez-Sobrido L, Topham DJ: Induction of CD8 T cell heterologous protection by a single dose of single-cycle infectious influenza virus. *J Virol* 2014, 88:12006–12016. [PubMed: 25100831]
 58. Baker SF, Guo H, Albrecht RA, Garcia-Sastre A, Topham DJ, Martinez-Sobrido L: Protection against lethal influenza with a viral mimic. *J Virol* 2013, 87:8591–8605. [PubMed: 23720727]
 59. Uraki R, Kiso M, Iwatsuki-Horimoto K, Fukuyama S, Takashita E, Ozawa M, Kawaoka Y: A novel bivalent vaccine based on a PB2-knockout influenza virus protects mice from pandemic H1N1 and highly pathogenic H5N1 virus challenges. *J Virol* 2013, 87:7874–7881. [PubMed: 23658445]
 60. Ozawa M, Victor ST, Taft AS, Yamada S, Li C, Hatta M, Das SC, Takashita E, Kakugawa S, Maher EA, et al.: Replication-incompetent influenza A viruses that stably express a foreign gene. *J Gen Virol* 2011, 92:2879–2888. [PubMed: 21880840]

61. Inagaki A, Goto H, Kakugawa S, Ozawa M, Kawaoka Y: Competitive incorporation of homologous gene segments of influenza A virus into virions. *J Virol* 2012, 86:10200–10202. [PubMed: 22740412]
62. Shinya K, Fujii Y, Ito H, Ito T, Kawaoka Y: Characterization of a neuraminidase-deficient influenza A virus as a potential gene delivery vector and a live vaccine. *J Virol* 2004, 78:3083–3088. [PubMed: 14990727]
63. Kobayashi H, Iwatsuki-Horimoto K, Kiso M, Uraki R, Ichiko Y, Takimoto T, Kawaoka Y: A replication-incompetent influenza virus bearing the HN glycoprotein of human parainfluenza virus as a bivalent vaccine. *Vaccine* 2013, 31:6239–6246. [PubMed: 24144478]
64. Victor ST, Watanabe S, Katsura H, Ozawa M, Kawaoka Y: A replication-incompetent PB2-knockout influenza A virus vaccine vector. *J Virol* 2012, 86:4123–4128. [PubMed: 22301144]
65. Fonseca W, Ozawa M, Hatta M, Orozco E, Martinez MB, Kawaoka Y: A recombinant influenza virus vaccine expressing the F protein of respiratory syncytial virus. *Arch Virol* 2014, 159:1067–1077. [PubMed: 24292020]
66. Baker SF, Nogales A, Finch C, Tuffy KM, Domm W, Perez DR, Topham DJ, Martinez-Sobrido L: Influenza A and B virus intertypic reassortment through compatible viral packaging signals. *J Virol* 2014, 88:10778–10791. [PubMed: 25008914]
67. Marsh GA, Hatami R, Palese P: Specific residues of the influenza A virus hemagglutinin viral RNA are important for efficient packaging into budding virions. *J Virol* 2007, 81:9727–9736. [PubMed: 17634232]
68. Bottini A, De SK, Baaten BJ, Wu B, Barile E, Soonthornvacharin S, Stebbins JL, Bradley LM, Chanda SK, Pellicchia M: Identification of small molecules that interfere with H1N1 influenza A viral replication. *ChemMedChem* 2012, 7:2227–2235. [PubMed: 23139022]
69. Si L, Xu H, Zhou X, Zhang Z, Tian Z, Wang Y, Wu Y, Zhang B, Niu Z, Zhang C, et al.: Generation of influenza A viruses as live but replication-incompetent virus vaccines. *Science* (80-) 2016, 354:1170–1173.

Figure 1. IAV virion structure and genome organization.

A) Virion structure: IAV particles contain a lipid membrane where the two major viral glycoproteins hemagglutinin (HA) and neuraminidase (NA), which are responsible for viral entry and release, respectively, and the ion channel matrix protein 2 (M2) are located. Below the viral lipid envelope is a layer composed of the matrix protein (M1) protein, which plays a role in virion assembly and budding; and the nuclear export protein (NEP), responsible for the nuclear export of the viral ribonucleoprotein (vRNP) complexes. Inside the viral particle are the vRNPs formed by the viral (v)RNA coated by the nucleoprotein (NP) and linked to the heterotrimeric RNA dependent RNA polymerase complex, containing the polymerase basic 2 and 1 (PB2 and PB1, respectively) and acidic (PA) proteins [2]. **B) Genome organization:** IAV contains eight vRNA segments (PB2, PB1, PA, HA, NP, NA, M, and NS) containing a coding region (gray) flanked at the 5' and 3' terminal ends by untranslated non-coding regions (NCRs, black lines) that work as promoters for viral replication and transcription. At the end of the 5' and 3' coding regions are located the packaging signals (white boxes) that are required for efficient encapsidation of the vRNPs into new viruses [3]. The IAV genome encodes for multiple viral proteins, with specific roles during the replication cycle of the virus [3].

Figure 2. Seasonal IIV and LAIV.

The strategy for generating current seasonal IAV vaccines involve the co-infection of embryonated chicken eggs with two viruses, the WHO candidate virus and the high-growth virus, for influenza inactivated vaccine (IIV, right); or the master donor virus (MDV) for live-attenuated influenza vaccine (LAIV, left). For the selection of the appropriate IIV or LAIV, antibodies against the HA and NA of the high-growth virus (IIV) or the MDV (LAIV) are used. Reassortant viruses containing the HA and NA genes from the WHO-recommended and the internal genes (PB2, PB1, PA, NP, M and NS) of the IIV or LAIV strain are amplified and purified for vaccine manufacture and distribution. In the case of IIV, viruses are previously inactivated before intramuscular (IM) inoculation. LAIV are administrated intranasally (IN).

Figure 3. Reverse genetics approaches for the recovery of recombinant IAVs.

A) Schematic representation of the ambisense plasmids to generate recombinant IAVs: IAV cDNAs are cloned into a bi-directional vector containing the human polymerase I promoter (hPol-I, black arrow) and the mouse Pol-I terminator (T, black box) sequences to drive the synthesis of the vRNAs. In opposite orientation to the polymerase I cassette are the polymerase II-dependent promoter (Pol-II, white arrow) and a polyadenylation sequence of the bovine growth hormone (BGH, white box) to allow the expression of viral proteins from the same viral cDNAs. **B) Plasmid-based reverse genetics to generate recombinant IAV:** FDA-approved cell lines for vaccine production are co-transfected with the eight (PB2, PB1, PA, HA, NP, NA, M, and NS) ambisense IAV plasmids. Recovered virus is amplified using fresh cells or embryonated chicken eggs that are used for vaccine fabrication.

Figure 4. Schematic representation of novel vaccines based on reverse genetics.

The different reverse genetics approaches described in this review to generate new LAIVs are shown. These include the use of new MDV (A), NS1 deficient and/or truncated viruses (B), IAV with altered NS1 and/or PA-X proteins (C), codon deoptimized (CD) viruses (D), viruses with rearranged genomes (E) or modified M and/or NS segments (F), single-cycle infectious (sci)IAVs (G) or premature termination codon (PTC) viruses (H). Wild-type segments are indicated in black. Modified segments are shown in gray.